

# Transcriptome analysis of *Arabidopsis* roots treated with signaling compounds: a focus on signal transduction, metabolic regulation and secretion

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## Summary

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Received: 28 December 2007  
Accepted: 25 February 2008

- Gene expression in response to signaling molecules has been well studied in the leaves of the model plant species *Arabidopsis thaliana*. However, knowledge of gene expression and metabolic regulation at the root level is limited.
- Here, the signaling compounds salicylic acid (SA), methyl jasmonate (MeJA) and nitric oxide (NO) were applied exogenously to induce various defense responses in roots, and their effect was studied using a combination of genomic, molecular and biochemical approaches. Genes involved in defense signaling/activation, cellular redox state, metabolism, transcription factors and membrane transport were altered in expression following treatment with SA, MeJA and NO.
- In addition, it was found that SA-, MeJA- and NO-elicited roots increased the root exudation of phytochemicals compared with the roots of nontreated control plants. Transport systems likely to be involved in the root exudation of phytochemicals, including the MATE, ABC, MFS, amino acid, sugar and inorganic solute transporters, showed altered expression profiles in response to treatments.
- Overall, significant differences were found in the signaling compound-elicited expression profiles of genes in roots vs those in leaves. These differences could be correlated to the underground nature of roots and their exposure to higher microbial inoculum rates under natural conditions.

**Key words:** *Arabidopsis*, defense response, methyl jasmonate (MeJA), nitric oxide (NO), root secretion, salicylic acid (SA), signaling molecules, transporters.

*New Phytologist* (2008) doi: 10.1111/j.1469-8137.2008.02458.x

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## Introduction

Signaling molecules such as salicylic acid (SA), methyl jasmonate (MeJA) and nitric oxide (NO) play key roles in plant defense responses. A considerable amount of evidence indicates that SA is a critical signaling molecule in the pathways

leading to local and systemic acquired resistance (SAR) and pathogenesis-related (PR) protein expression (Delaney *et al.*, 1994; Kunkel & Brooks, 2002; Shah, 2003). A recent report demonstrates that the SA derivative methyl salicylate is a critical mobile signal for plant SAR (Park *et al.*, 2007). In addition, numerous defense-related genes and transcription factors are regulated by SA response (Uknes *et al.*, 1992; He *et al.*, 1999; Chen & Chen, 2002; Wagner *et al.*, 2002).

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Jasmonic acid (JA) and its derivative MeJA also mediate plant defense against insects, and appear to mobilize antimicrobial defense responses predominantly effective against necrotrophic pathogens, while the SA-mediated defense responses are effective against biotrophic fungi, bacteria and viruses (Thomma *et al.*, 2001; Murphy & Carr, 2002). Jasmonic acid is known to regulate uniquely PDF1.2, thionin and several other antimicrobial proteins (Penninckx *et al.*, 1998; Reymond & Farmer, 1998). Jasmonic acid also regulates the genes involved in JA biosynthesis during the defense response (Bell & Mullet, 1993; Melan *et al.*, 1993). Examinations of the cross-talk between the SA and JA pathways have revealed that it is complex and antagonistic (Peña-Cortés *et al.*, 1993; Niki *et al.*, 1998; Spoel *et al.*, 2003). NO was first identified in mammalian systems as a signaling molecule that regulated the immune and nervous systems (Wink & Mitchell, 1998). Although less is known about the role of NO in plants, accumulating evidence shows that NO is involved in various aspects of plant growth (Beligni & Lamattina, 2000; Leshem & Pinchasov, 2000), protection against UV and herbicides (Beligni & Lamattina, 1999; Mackerness *et al.*, 2001), and in defense responses (Klessig *et al.*, 2000; Wendehenne *et al.*, 2001). NO also regulates disease-resistance genes, transcription factors and signal transduction factors (Parani *et al.*, 2004). Several lines of evidence indicate an interrelationship between SA and NO signaling pathways, and also between NO and JA signaling pathways (Grün *et al.*, 2006).

All three signaling molecules stimulate the production of a wide range of secondary metabolites in plants (Zhao *et al.*, 2005), mostly related to defense responses. SA upregulates genes involved in glucosinolate biosynthesis in tobacco cell cultures (Taguchi *et al.*, 2001) and indole alkaloids in *Catharathus roseus* cell cultures (Zhao *et al.*, 2000). NO also induces secondary metabolites in plants, including phytoalexin production in soybeans (Modolo *et al.*, 2002) and catharanthine production in *C. roseus* cell cultures (Zhao *et al.*, 2005). Jasmonic acid and its analogues (MeJA) induce biosynthesis of secondary metabolites, including terpenoids, flavanoids, alkaloids and phenylpropanoids (Tamogami *et al.*, 1997; Brader *et al.*, 2001; Farmer *et al.*, 2003). All these studies (including transcriptional profiling of gene expressions and secondary metabolites) related to the effect of signaling molecules have been performed on the leaves of plants or cell cultures, and to the best of our knowledge nothing is known about the gene cascades that these signaling compounds initiate in plant roots.

The soil and the rhizosphere are environments with high pathogen inoculum loads (Rouatt & Katznelson, 1960; Rouatt *et al.*, 1960). Thus roots are exposed to an array of microbes in the rhizosphere, and must interact and defend according to the type of biotic stimulus (Bais *et al.*, 2004, 2006). In addition, it has been shown that root exudates play a role in defense responses against microbes (Bais *et al.*, 2006), and exogenously applied signaling compounds can result in quan-

titative and qualitative changes in the composition of exudates (Gleba *et al.*, 1999; Walker *et al.*, 2003). Moreover, SA, MeJA and NO act as systemic signals (Li *et al.*, 2002; Stohr & Ullrich, 2002; Park *et al.*, 2007) in defense responses, and it can probably be assumed that the signals transfer from shoots to the root system and thus alter the root exudation of compounds to defend against pathogen attack. It has been demonstrated that exogenous MeJA can be transported readily in the phloem (Zhang & Baldwin, 1997), and is also found in the medium of cultured plant cells (Parchmann *et al.*, 1997). Similarly, SA was also found in the medium of cultured plant cells, and the secretion mode was Ca<sup>2+</sup>-dependent and Ca<sup>2+</sup>-independent (Chen *et al.*, 2001). NO is produced by the plant and by rhizosphere bacterial nitrification and denitrification, and can be found in the apoplastic space of plant roots (Stohr & Ullrich, 2002). We designed this study to explore whether the defense responses and other gene cascades in response to signaling events characterized for leaves in *Arabidopsis* are similar to those present in roots in response to signaling compounds.

In this study, we treated the roots of *Arabidopsis* with signaling compounds and analysed the global patterns of gene expression. Changes in the root exudation of phytochemicals were also analysed in this study and subsequently correlated with gene expression profiles. We found that all three signaling molecules alter significantly < 1% of gene expression in roots, and include disease resistance genes, transcription factors, transporters, signal transduction genes and secondary metabolic pathway genes. Interestingly, we found only a limited overlap between the transcriptome profiles for each of the three treatments. In addition, we discuss the differential response of these signaling molecules between roots and leaves related to gene expression profiles. Finally, because phytochemicals are synthesized in the roots before secretion, we also discuss the regulation of metabolic pathways and transport systems.

## Materials and Methods

### Plant material and growth conditions

Wild-type *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia (Col-0) seeds (Lehle Seeds, Round Rock, TX, USA) were surface-sterilized using sodium hypochlorite (3% v/v) for 2 min followed by three rinses in sterile distilled water, and plated on Murashige and Skoog (MS) (Murashige & Skoog, 1962) salts supplemented with 3% sucrose and 0.7% bactoagar in Petri dishes. Plates were incubated in a growth chamber (Percival Scientific, Perry, IO, USA) at 25°C, with a photoperiod of 16 h light/8 h dark for germination. To collect root exudates, 7-d-old seedlings were transferred to six-well culture plates (Fischer Co., Hampton, NH, USA) each containing 5 ml of liquid MS (MS basal salts supplemented with 3% sucrose), incubated on an orbital shaker at 90 rpm and illuminated under cool white fluorescent light (45 μmol m<sup>-2</sup> s<sup>-1</sup>) with a photoperiod of 16 h light/8 h dark at 25°C.

## Treatment of *Arabidopsis thaliana* plants with signaling compounds

According to the methodology described previously (Loyola-Vargas *et al.*, 2007; Badri *et al.*, 2008), when plants were 18 d old the roots were washed with sterile water to remove the surface-adhered exudates, and transferred to new six-well plates containing 5 ml MS liquid media containing 250  $\mu\text{M}$  of MeJA, SA or NO donor sodium nitroprusside (Guo *et al.*, 2003), incubated on an orbital shaker at 90 rpm and illuminated under cool white fluorescent light (45  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) with a photoperiod of 16 h light/8 h dark at 25°C. The roots and root exudates present in the media were collected 3 and 6 h post-treatment.

## RNA isolation and microarray experiments

Total RNA was isolated from frozen root tissues collected from different treatments of SA, MeJA and NO and the control 3 h post-treatment using the RNeasy midi kit (Qiagen Inc., Germantown, MD, USA) and quantified using a Nanodrop ND-1000 Spectrophotometer. RNA integrity was checked on a formamide denaturing agarose gel and also estimated by 28S/18S ratio using an Agilent 2100 Bioanalyser (Palo Alto, CA, USA). The microarray analysis was performed at the Yale University Microarray facility center (Keck Biotechnology Resource Laboratory, CT, USA). For each replicate, 2.5  $\mu\text{g}$  total RNA was used to prepare cDNA using Superscript Reverse Transcriptase III (Invitrogen, CA, USA), and this cDNA was used for the preparation of Cy5-labelled and Cy3-labelled probes using 3D Array900 kit (Genisphere, Hatfield, PA, USA) and Advalytix Array Booster DNA Microarray Incubator (Olympus America Inc., Concord, MA, USA). Microarray analysis was performed using the *Arabidopsis* Genome Oligoset Version 1.0 (OAR27K) chip which contains 26 090 70 mers representing 26 090 genes (nearly whole-genome coverage) and predicted ORFs obtained from the Unigene Build 4 developed at the National Center of Biotechnology Information ([www.ncbi.nlm.nih.gov/UniGene](http://www.ncbi.nlm.nih.gov/UniGene)). The chip also includes 12 unique positive and negative controls each provided in 16 replicates in a 384-well plate. To assess the reproducibility of microarray data set results, we performed the microarray experiment twice with the subset of two samples each pooling three independent biological replicates.

## Microarray data analysis

After hybridization, the slides were scanned immediately using a Genisphere Array Scanner and transformed into tif images for subsequent data analysis. The background-subtracted spot intensities were measured using SPOTFINDER ver. 3.0 and normalized by the Lowess method. Genes showing a signal value < 600 in both Cy3 and Cy5 channels of the control and treatment were not considered for the

analysis. A significant analysis test (unpaired *t*-test with Benjamini–Hochberg false discovery rate correction) was performed to test the equality of the mean signal values between the treatments. Fold change was then calculated as the simple ratio of overall signal values between the control and treatment. Genes with *P* values  $\leq 0.05$  and up- or downregulated at least twofold in all three treatments were considered to be significantly differentially expressed.

## Quantitative RT-PCR

Two micrograms of purified total RNA from root tissues collected 3 h after treatment with elicitors was reverse-transcribed using Superscript III RT and poly(T) primer at 42°C for 1 h (Invitrogen) according to the manufacturer's instructions. The reaction product was diluted to a concentration of 50  $\text{ng } \mu\text{l}^{-1}$  and 1  $\mu\text{l}$  was used for each PCR reaction. The reaction mix (20  $\mu\text{l}$ ) contained 0.4  $\mu\text{mol}$  of each gene-specific primer, 200  $\mu\text{mol}$  dNTPs, 1 $\times$  reaction buffer and 1 U Taq DNA polymerase (Takara, Madison, WI, USA). PCR included 29 cycles of 94°C for 30 s, 52°C for 30 s and 72°C for 2 min in an Applied Biosystems (Foster City, CA, USA) thermal cycler (GeneAmp PCR system 2700). Actin primers were used as a control to determine the uniformity of the concentration of cDNA. The gene-specific primers used for RT-PCR assays are listed in Table S1 in Supplementary material. The quantification of fold induction was determined by measuring the band intensities using Adobe PHOTOSHOP ver. 6.0 after normalization with control actin. The PCR was carried out three times with three independent biological replicates. A significant analysis test (unpaired *t*-test with Benjamini–Hochberg false discovery rate correction) was performed to test the equality of the mean signal values between treatments.

## Root exudate extraction

After the different treatments, the collected liquid medium containing the root exudates was filtered using nylon filters of pore size 0.45  $\mu\text{m}$  (Millipore, Jaffrey, NH, USA) to remove root sheathing, broken cells and root border-like cells. After filtration, the liquid was concentrated by freeze-drying (Labconco, Kansas City, MO, USA) to remove water, and the concentrate was dissolved in 5 ml double-distilled water. The concentrate was partitioned three times with 5 ml ethyl acetate (Fisher Scientific, Fair Lawn, NJ, USA). The ethyl acetate fractions were pooled and dried under air flux. The dried concentrate was again dissolved in 1 ml absolute methanol (Fisher Scientific) and analysed by HPLC-mass spectrometry. The experiment was repeated twice with three replicates each.

## HPLC and mass spectrometry analyses

The phytochemicals extracted from liquid media were chromatographed by gradient elution on a 150  $\times$  4.6 mm

reverse-phase, C18 column (Dionex, Sunnyvale, CA, USA). The chromatographic system (Dionex) consisted of two P680 pumps connected to an AS1-100 automated sample injector and detected at 280 nm with a UV-visible detector. Mass determination of the peaks was analysed using an MSQ-MS detector system (Thermo Electron Co., Waltham, MA, USA). A gradient was applied for all separations with a flow rate of 0.7 ml min<sup>-1</sup>. The gradient was as follows: 0–3 min, 90% water and 10% methanol; 3–43 min, 10–90% (v/v) methanol and 90–10% (v/v) water; 43–51 min, 90% (v/v) methanol and 10% (v/v) water.

#### Isolation and identification of phytochemicals present in the root exudates

Total liquid medium from 25-d growth of *c.* 8000 seedlings was collected and extracted three times with an equal volume of ethyl acetate; the ethyl acetate layer was evaporated to dryness to leave 800 mg of gummy residue. This residue was chromatographed through a Sephadex LH-20 column (Pharmacia, Piscataway, NJ, USA) to yield seven fractions: fraction 1 (nothing), 2 (1 mg), 3 (90 mg), 4 (500 mg), 5 (30 mg), 6 (8 mg) and 7 (17 mg). The major fraction (fraction 4) was composed mainly of sugar or sugar-like compounds. Fractions 5 and 6 were combined and purified by HPLC on a C-18 column eluted with 0.1% acetic acid in water with increasing amounts of methanol added. The eluate from 0 to 15 min was collected and then six collections were made successively every 5 min. Each 5-min collection was evaporated to dryness and analysed by proton nuclear magnetic resonance (NMR), liquid chromatography–mass spectrometry (LCMS) (low resolution) and finally by LCMS (high resolution), with the following results.

The 25–30-min collection, after rechromatography, yielded kaempferol-3, 7-*O*-bisrhamnoside (KRR) and kaempferol-3-*O*-β-D-glucopyranoside-7-*O*-α-L-rhamnoside (KGR). These compounds were characterized by use of extensive proton and C-13 NMR spectroscopy in comparison with literature data for KRR (Veit & Pauli, 1999; Routaboul *et al.*, 2006) and KGR (Veit & Pauli, 1999; Routaboul *et al.*, 2006). The 30–35-min collection, after rechromatography, yielded methyl indolyl-3-carboxylate (MIC) and kaempferol-3-*O*-α-L-rhamnoside (KR) as well as other compounds currently under investigation. MIC had NMR spectra identical to a standard commercial sample (Aldrich Chemical Co., Milwaukee, WI, USA), while KR was characterized by NMR spectra in comparison with literature data (Routaboul *et al.*, 2006). In the cases of KRR and KGR, insufficient material was obtained from the root exudates for complete spectral characterization, but these compounds were available in greater quantities from leaf material by a similar isolation scheme. The two flavonoid diglycosides (KRR and KGR) have previously been found in other parts of *Arabidopsis*, but have not been identified from root exudates. KR and MIC have been

reported once previously from *Arabidopsis* root exudates (Narasimhan *et al.*, 2003) (Fig. S1).

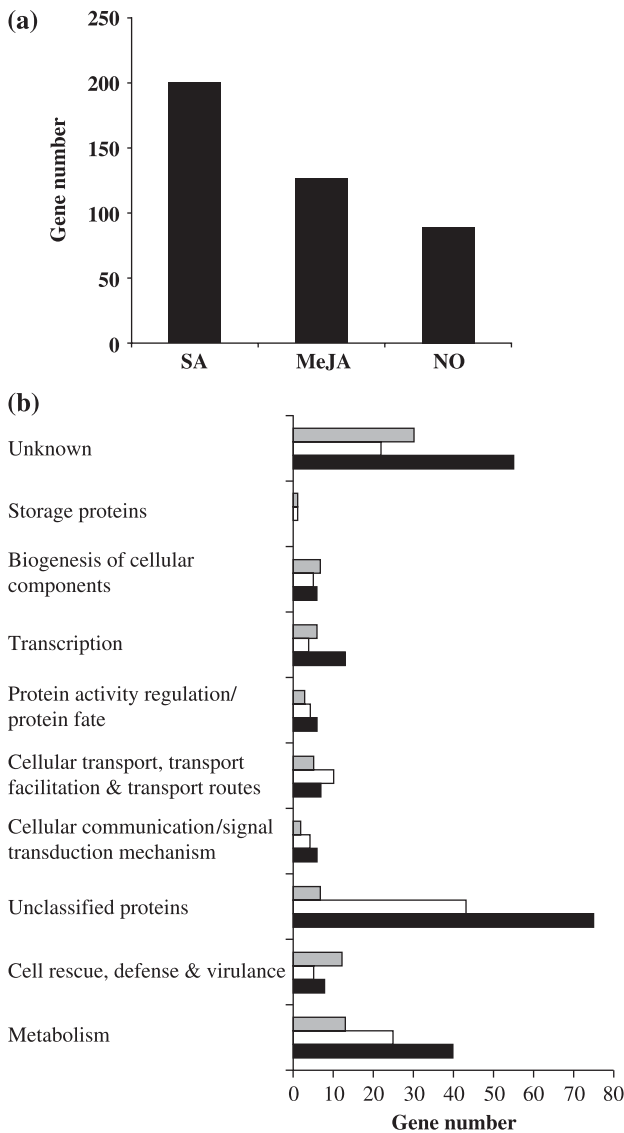
#### Principal components analyses

Principal components analysis (PCA) was performed using the average value per treatment and per peak to identify the most important variables in the expression of treatment and peak differentiation, and to reduce the dimensionality of the variability to the lowest possible number of PCs. In these analyses, the matrix of correlations was used. The treatments were represented graphically to identify their grouping pattern. Euclidian distances between pairs of treatments and peaks were determined and a cluster analysis of these was conducted using NTSYSpc ver. 2.01 for Windows (<http://www.exetersoftware.com/cat/ntsyspc/ntsyspc.html>) with the unweighted pair-group method with arithmetical averages (UPGMA) method. The linear transformation of our phytochemical data performed by this method generated a new set of seven independent variables, which are described by their latent roots and vectors. As there are no tests to evaluate the significance of latent roots, we chose to follow the criterion established by Kaiser (1960), which adapt very well to the purpose of this analysis. This criterion is based on the selection of principal components with latent roots > 1. According to this criterion, the first three components qualify, accounting for > 69% of total variation, giving a clear idea of the structure underlying the quantitative variables analysed.

## Results

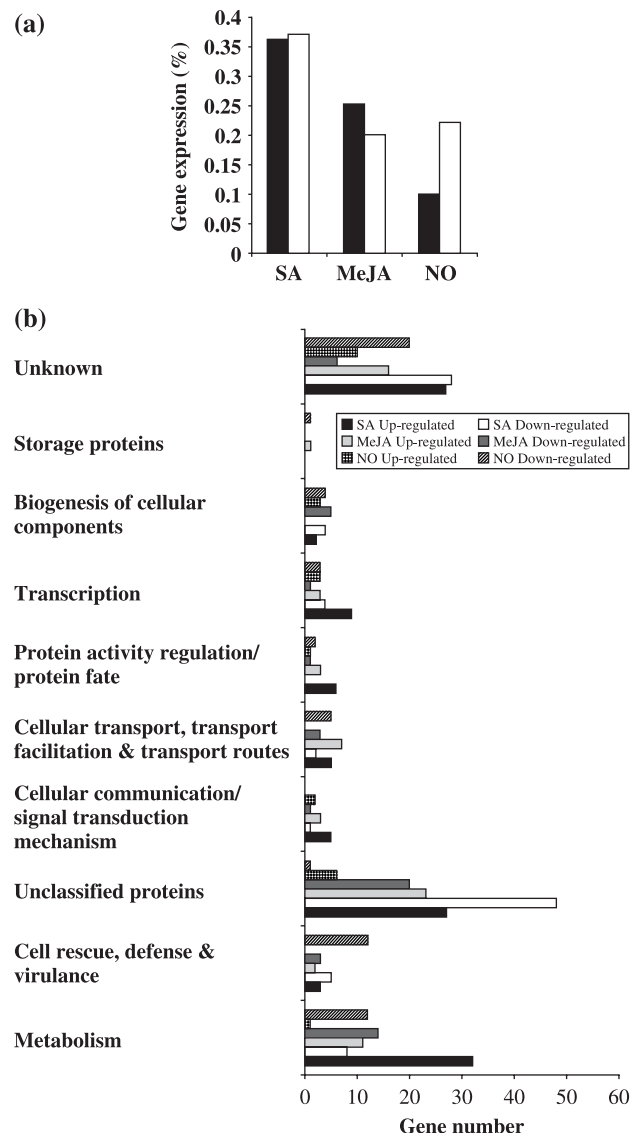
#### Overall gene regulation in response to SA, MeJA and NO

A whole-genome approach was pursued using a 70-mer oligo microarray representing 26 090 predicted unigenes of *Arabidopsis* to characterize root-specific genes elicited by the signaling compounds. Pairwise comparisons were made between the control and each treatment independently and between the treatments. Examination of the expression ratios of genes indicated that a relatively small portion of the genome was differentially expressed in each signaling molecule treatment. We found that 199 (0.76%), 125 (0.47%) and 87 genes (0.33%) were differentially expressed more than twofold with a *P* value < 0.05 in SA, MeJA and NO treatments, respectively (Fig. 1a). Transcript levels of < 1% of the *Arabidopsis* genes were significantly altered in each of these treatments. The genes that were altered in each signaling molecule treatment were distributed into 10 different functional categories (Fig. 1b) based on FunCat assignments available through the MIPS *A. thaliana* database (<http://mips.gsf.de/proj/plant/jsf/athal/index.jsp>), BLAST searches and literature reports. The largest categories identified were 'unknown' and 'unclassified proteins' (Fig. 1b), indicating there are



**Fig. 1** (a) Differentially expressed genes in *Arabidopsis* roots treated with the signal molecules salicylic acid (SA), methyl jasmonate (MeJA) and nitric oxide (NO) at 250  $\mu\text{M}$  concentration after 3 h compared with control in microarray analysis. (b) Differentially expressed genes in microarray analysis were assigned to various functional categories based on the FunCat scheme devised by the Munich Information Center for Protein Sequences for the three signaling molecule treatments. The differentially expressed genes represented are statistically significant at a  $P < 0.05$  and show more than twofold induction. NO, grey bars; MeJA, white bars; SA, black bars.

many novel genes altered by treatment with the signaling compounds. Also, the functional categories 'metabolism', 'transcription' and 'cellular transport, transport facilitation and transport routes' were differentially expressed upon signaling molecule treatment (Fig. 1b). Some of the genes involved in transport will be discussed specifically later. The numbers of genes induced or repressed in all three elicitor treatments were similar in number except for the



**Fig. 2** (a) Percentage of genes significantly upregulated (black bars) and downregulated (white bars) in *Arabidopsis* roots treated with the signal molecules salicylic acid (SA), methyl jasmonate (MeJA) and nitric oxide (NO) at 250  $\mu\text{M}$  concentration after 3 h compared with control. (b) The number of genes significantly upregulated and downregulated on three signaling molecule treatments (SA, MeJA and NO) was assigned to functional categories based on the FunCat scheme devised by the Munich Information Center for Protein Sequences. The differentially expressed genes represented are statistically significant at  $P < 0.05$  and show more than twofold induction.

NO treatment, which had a high number of repressed genes and few induced genes (Fig. 2a). In particular, SA induced a significant number of genes involved in the functional category 'metabolism'. In the MeJA treatment, the number of genes induced or repressed related to metabolism is apparently similar, but in the NO treatment the number of genes repressed is higher than those induced (Fig. 2b). The trend

looks similar for the other functional categories 'transcription' and 'cellular transport' for SA and NO treatments. In addition to these functional categories, there are also genes differentially expressed (induced or repressed) in the other functional categories 'cell rescue, defense and virulence', 'cellular communication and signal transduction mechanism'.

#### Genes significantly regulated in defense signaling/activation

Regulation of genes encoding receptor-like serine/threonine kinases was found in response to SA and MeJA, but not in response to NO. Interestingly, the receptor-like serine/threonine kinases regulated by SA and MeJA are different, with one upregulated (At3g53030) by MeJA and one downregulated (At1g29750) by SA. In addition to these receptor-like kinases, other kinases (mitogen-activated protein kinases) were found to be differentially regulated in response to various signaling molecules. We also found other defense signaling/activation-related genes belonging to the members of the GTP-binding protein family, the zinc finger protein family, and the calmodulin-binding protein family, and transcription factors including members of the WRKY and AP2 families (Tables S2–S4). Our analyses showed that the GTP-binding proteins are upregulated by SA (At4g17530 and At5g03520) and MeJA (At4g35860) treatments but not by NO treatment. Similarly, MeJA and NO treatments showed downregulation of calmodulin-binding proteins but no differential expression of these genes was observed under SA treatment. The transcription factors belonging to the families AP2 are upregulated by SA and NO treatments but not by MeJA. The WRKY family transcription factor was observed only to be upregulated by SA and not by MeJA or NO treatments (Tables S2–S4).

#### Genes involved in antimicrobial activities

We found only a limited number of genes belonging to the family of antimicrobial proteins that were differentially regulated in response to the three signaling molecules. The PR gene families, such as glucanases, were upregulated by SA treatment but downregulated by MeJA treatment. Interestingly, the glucanases differentially regulated by SA and MeJA belonged to different subfamilies. In addition, we found some proteases upregulated only by MeJA treatment, but not SA or NO treatments. Other antimicrobial proteins were observed to be downregulated by NO treatment (Tables S2–S4).

#### Genes involved in regulation of cellular redox state

A considerable number of genes related to the regulation of cellular oxidative status were differentially regulated in all three treatments. All three treatments repressed peroxidase genes, but the specific genes repressed were different, and

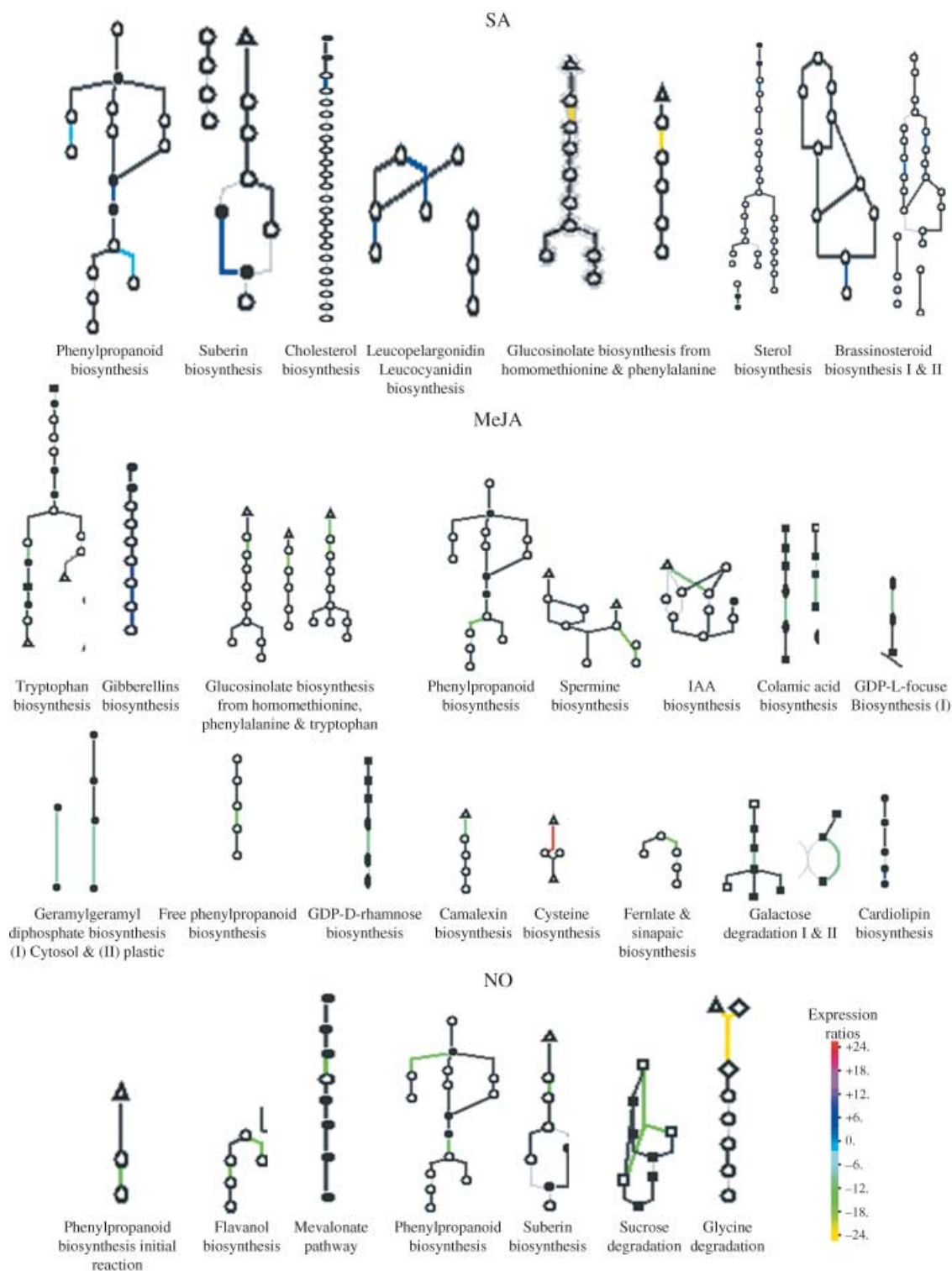
specific to each signaling molecule response. SA and MeJA treatments regulated other genes related to oxidative status, including glutaredoxin, thioredoxin and glutathione *S*-transferase, but none of these genes was differentially regulated by NO treatment. Interestingly, MeJA repressed the thioredoxin and glutathione *S*-transferase genes and SA induced the glutaredoxin gene. Taken together, all three signaling molecules employed a different and specific signaling mechanism to regulate the oxidative state of the root cells (Tables S2–S4).

#### Effect of three signaling molecule treatments on metabolic pathways

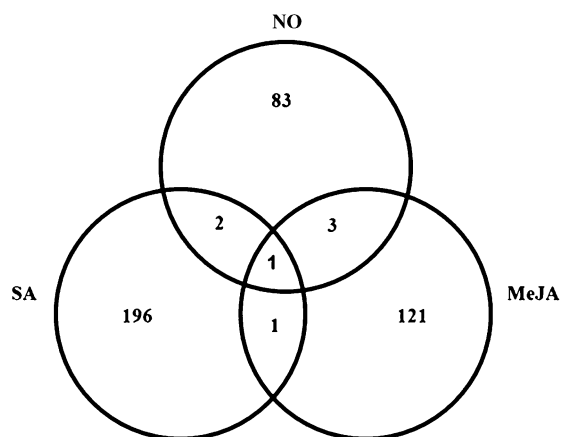
We followed the standardized AraCyc-defined metabolic pathways (Mueller *et al.*, 2003), which currently include 1759 *Arabidopsis* enzyme genes, to identify genes in each pathway. To examine similarities and differences among the three signaling molecule treatments, entire biosynthetic pathways were analysed. We found that metabolic pathways were affected significantly by all three treatments at the root level (Fig. 3). The three signaling molecule treatments affected different metabolic pathways with a limited overlap. For example, the phenylpropanoid biosynthesis pathway was affected in all three treatments, but the regulated steps (specific genes repressed) were different in each treatment. By contrast, glucosinolate biosynthesis from the homomethionine and phenylalanine pathways was affected by the salicylic acid and methyl jasmonate treatments, but the regulated steps (genes repressed) were similar in each treatment. The suberin biosynthesis pathway was affected by SA and NO treatment, but the regulated steps were different in each treatment. In addition, under SA treatment the affected step was induced, but under NO treatment the affected step was repressed. Treatment with SA caused significant induction of metabolic steps in the pathways of suberin biosynthesis, cholesterol biosynthesis, leucopelargonidin and leucocyanidin biosynthesis, sterol biosynthesis and brassinosteroid biosynthesis, and repression of metabolic steps in the pathways of phenylpropanoid biosynthesis and glucosinolate biosynthesis from homomethionine and phenylalanine. Similarly, NO treatment caused significant repression of metabolic steps in the major pathways of phenylpropanoid biosynthesis and flavanol biosynthesis, and also in the sucrose and glycine degradation pathways. On the whole, MeJA altered the regulation of more metabolic pathways than the other two signaling molecule treatments. Interestingly, hormone biosynthesis genes were barely regulated in all three treatments, except MeJA, which showed significant induction and repression of metabolic steps in the pathways of gibberellins and indole acetic acid, respectively.

#### Genes involved in miscellaneous gene families

Genes outside the functional groupings described previously that were notably regulated by SA included heat-shock



**Fig. 3** Diagram of representative metabolic pathways regulated by the three elicitor treatments at the root level. Each pathway is shown as a glyph consisting of nodes and lines, which represent the metabolites and reactions, respectively. Expression-level change of each reaction is shown in a color relative to the expression level, as indicated in the color scale bar. SA, salicylic acid; MeJA, methyl jasmonate; NO, nitric oxide. Triangle, amino acids; square, carbohydrates; diamond, proteins; open circle, others; closed circle, phosphorylated.

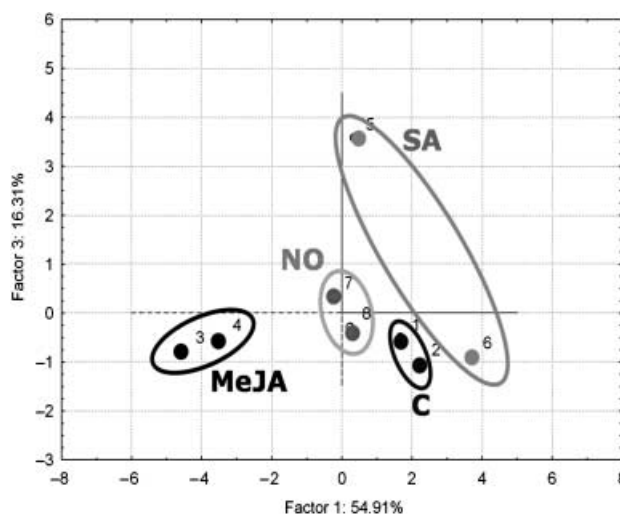


**Fig. 4** Venn diagram showing the number of significantly differentially expressed genes and the shared genes in all three signaling molecule treatments: salicylic acid (SA), methyl jasmonate (MeJA) and nitric oxide (NO).

proteins/chaperones, pectin methylesterase, kelch repeat protein, monooxygenases, glycosyl hydrolase, epithiospecifier protein and proline oxidase (Table S2). Similarly, genes outside major groups particularly regulated by MeJA include those involved in heat-shock proteins/chaperones, chlorophyll *a*-*b*-binding protein, the two-component regulator gene histidine kinase, no apical meristem (NAM) protein, auxin-induced protein, beta-glucosidase, acid phosphatases, amino acid permeases, kunitz family protein, MADS box family protein, constans-like protein and myrosinase-binding protein (Table S3). In response to NO, the genes outside the major group include those for heat-shock proteins/chaperones, leucine zipper protein, chlorophyll *a*-*b*-binding protein, cytokinin signaling pathway response regulator and coronatine-induced protein (Table S4).

#### Clear specificities for each treatment at the transcriptional level

A rather limited gene overlap was found among these three treatments, as shown in a Venn diagram (Fig. 4). There was only one gene (a transcription factor, At3g29035) upregulated in all three treatments. There are slight overlaps: three genes (sucrose transporter, At1g22710; aspartyl protease, At3g61820; calmodulin-binding protein At3g51920) were downregulated by MeJA and NO treatments. Similarly, only one gene (a transcription factor, At1g43160) was upregulated and only one gene (leucine-zipper protein, At1g69780) was downregulated by NO and SA treatments, respectively. An additional gene (cytochrome *P*<sub>450</sub> monooxygenase, At4g13770) was downregulated by SA and MeJA treatments. These results suggest that distinct genome reprogramming is specifically involved in each treatment at the root level. There was no overlap in other functional categories (transporters, secondary metabolism, etc.).



**Fig. 5** Principal components analysis shows differences between the elicited and control samples.

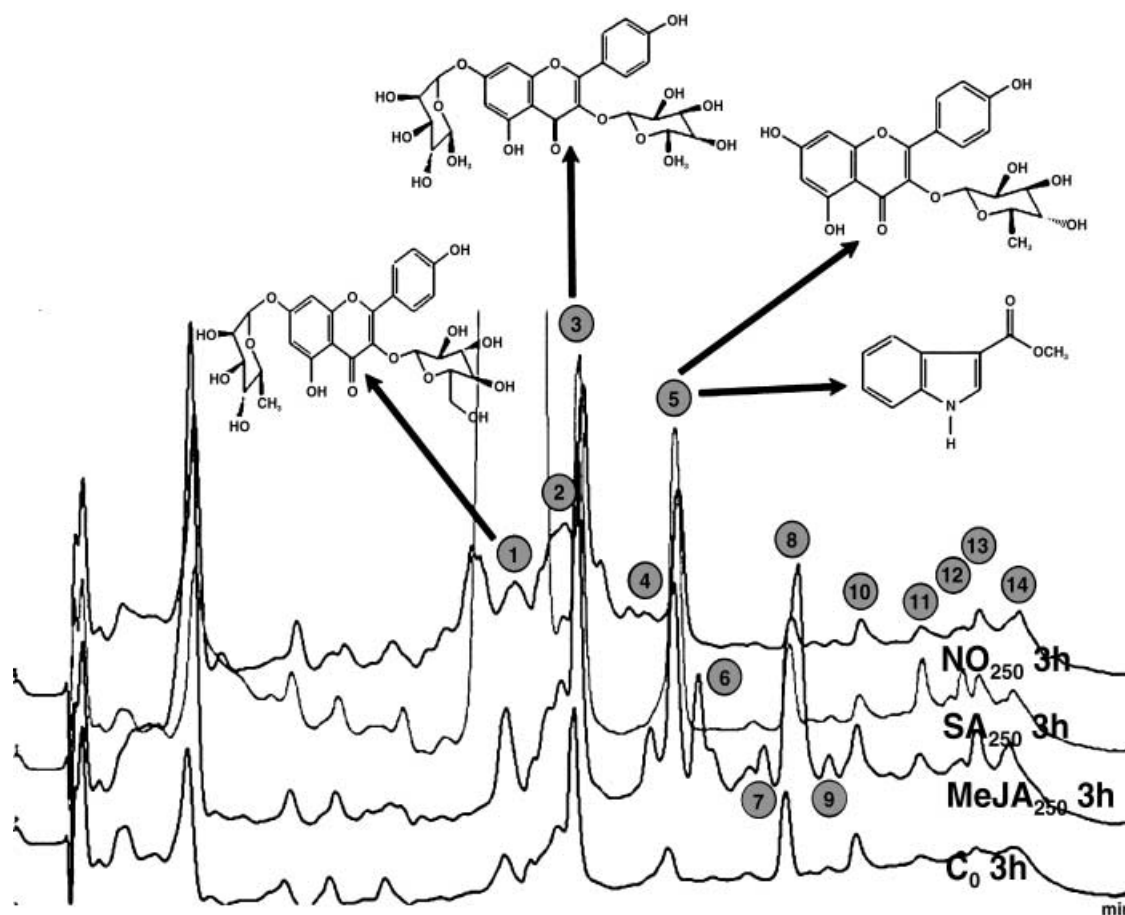
#### Verification of microarray data by quantitative RT-PCR

Several transcripts of selected genes either upregulated or downregulated based on microarray analysis were quantified independently by quantitative RT-PCR (Tables S2–S4). They include an NAC transcription factor (At3g29035), MFS transporters (At5g13750, At5g64500), MATE transporters (At5g17700, At3g23560) and secondary metabolism-related genes including spermine synthase (At5g19530), ferulate 5-hydroxylase (At4g36220), flavanol synthases (At5g05600, At5g08640) and *O*-methyl transferase (At1g21120). The quantitative RT-PCR results of the 12 genes showed similar differential expression patterns to those obtained by microarray analysis (Fig. S2).

#### Elicitation of roots and the effect on root exudation

We also elicited the roots with MeJA, SA and NO to determine if those changes affected the production and secretion of secondary metabolites by roots (Xu *et al.*, 2005; Grün *et al.*, 2006; Halim *et al.*, 2006). Our combined HPLC-MS methodology revealed clear differences in the profiles of phytochemicals secreted by the roots treated with the elicitors compared with those of the nontreated controls. Principal components analysis determined that the three treatments segregated from the control sample and were clearly distinguishable along the first PC axis (Fig. 5). The two time points taken for each treatment were similar, with the exception of SA treatments, in which the profiles for 3 and 6 h were significantly different.

In general, there were differences in the number of compounds induced by the specific treatments at two time points (3 and 6 h) compared with the control (Fig. 6; Fig. S3). The



**Fig 6** Chromatogram of representative HPLC/MS *Arabidopsis thaliana* plant root exudate profiles in the presence of 250  $\mu\text{M}$  of elicitor after 3 h treatment. MeJA, plants elicited with MeJA; SA, plants elicited with SA; NO, plants elicited with NO; CO, plants with no elicitors. The compound structures identified by NMR from the respective peak numbers were represented. Kaempferol-3-*O*- $\beta$ -D-glucopyranoside-7-*O*- $\alpha$ -L-rhamnoside (MM = 594; peak 1); Kaempferol-3,7-*O*-bisrhamnoside (MM = 578; peak 3); Methyl indolyl-3-carboxylate (MM = 175; peak 5) and kaempferol-3-*O*- $\alpha$ -L-rhamnoside (MM = 432; peak 5).

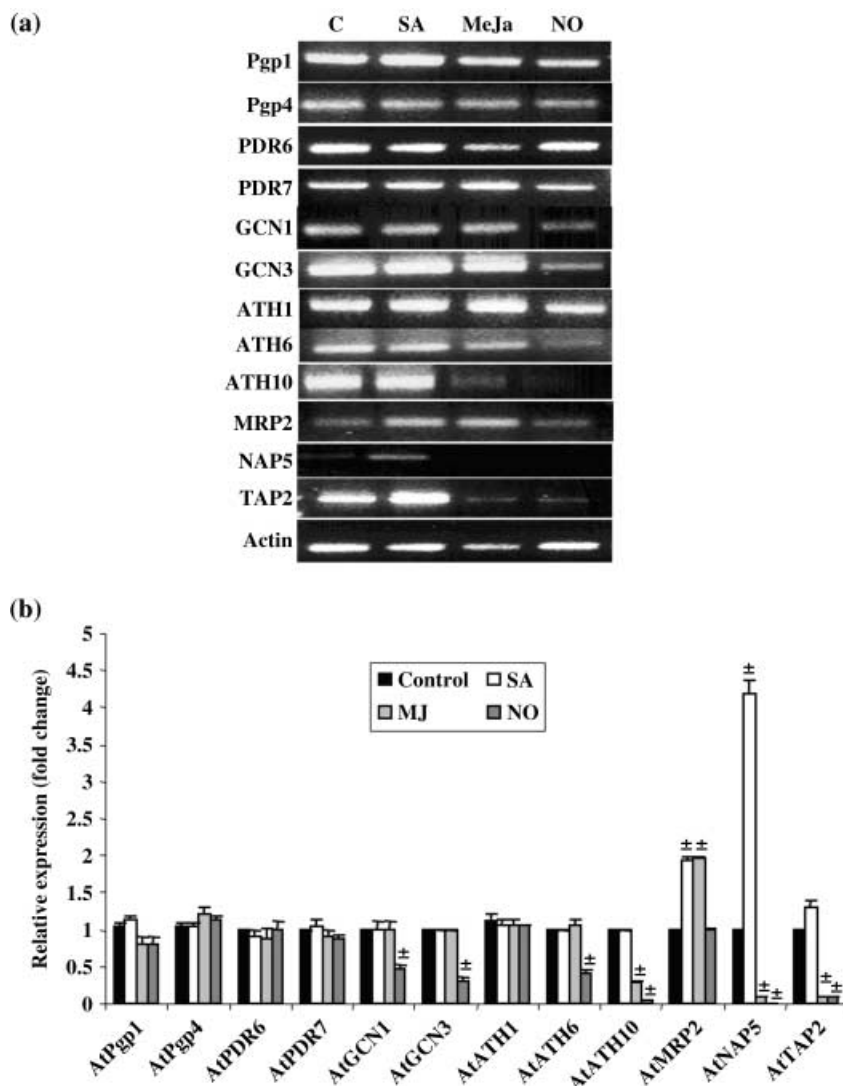
treatment with MeJA produced the biggest increase in the concentration of phytochemicals in the exudates compared with the other treatments. Eight phytochemicals increased more than twofold after 3 h treatment with MeJA, while only two compounds increased twofold with the SA and NO treatments (Fig. S4a,b). The presence of specific compounds in the root exudates was very dynamic; for instance, peak 9 (MM = 312) is increased by NO and peak 12 (MM = 326) by SA at 3 h, but both peaks are absent after 6 h incubation in all treatments, including the controls (Fig. S4b).

Some of the peaks were purified and identified by NMR and their secretion patterns were monitored in all three treatments. Kaempferol-3-*O*- $\beta$ -D-glucopyranoside-7-*O*- $\alpha$ -L-rhamnoside (MM = 594; peak 1) was induced only by MeJA but was absent in SA treatments at both time points. Kaempferol-3,7-*O*-bisrhamnoside (MM = 578; peak 3) was increased in all treatments at 3 h, but decreased only in the SA treatment at 6 h. Methyl indolyl-3-carboxylate (MM = 175;

peak 5) and kaempferol-3-*O*- $\alpha$ -L-rhamnoside (MM = 432; peak 5) increased in all treatments at both time points.

#### Genes involved in membrane transport

Elicitation of roots with signaling molecules increased the amount of root-exuded phytochemicals, and the exudation of specific compounds was increased by specific elicitation by a signaling molecule. We assumed that specific induction of transporters might be involved in the transport of specific compounds outside the root cells, as observed after the elicitation of roots. A considerable number of transporters were differentially regulated by all three signaling molecule treatments. SA and MeJA treatments showed both up- and downregulation of transporters, but NO treatment showed only downregulation of transporters. These transporters include members of the MATE, MFS and AAA-type ATPase families, sugar transporters and amino acid transporters (Tables



**Fig. 7** Analysis of the expression of ABC transporter genes in roots of *Arabidopsis* on signaling molecule treatment. (a) Gel pictures showing the expression pattern of 12 ABC transporter genes in *Arabidopsis* roots. DNAase-treated RNA from roots was used to prepare first-strand cDNA. An equal quantity of first-strand cDNA was used in PCR with primers specific to each gene. Sequences of forward and reverse primers used in PCR are presented in Table S1. An equal quantity of template in each reaction was verified by amplifying a constitutively expressed actin. The name of the ABC transporter gene is shown on the left of each panel. The different treatments are shown in the top panel of the gel picture. (b) Quantification of gene expression. Each gene's expression under a particular treatment is quantified and normalized in comparison with actin levels and presented as fold values. Asterisks indicate that a value is statistically significant ( $P < 0.05$ ) compared with its respective control. Data are shown as mean of three replicates. Error bars,  $\pm$ SD; C, control; SA, salicylic acid; MEJA, methyl jasmonate; NO, nitric oxide.

S2–S4). SA and MeJA induced more transporter genes than they repressed. SA differentially regulated MFS, MATE, ammonium, sulfate, potassium and calcium transporters, and MeJA differentially regulated MATE, an  $H^+$  ATPase pump, a sugar transporter and a metal transporter. Interestingly, only MeJA and NO differentially regulated the expression of sugar and amino acid transporters; SA treatment had no effect. Surprisingly, we did not observe any ABC transporters significantly differentially regulated (more than twofold up- or downregulated) in any of the three signaling molecule treatments in our transcriptome analysis.

In order to improve the resolution of the analysis and to determine if ABC transporter genes are induced on treatment with signaling compounds, we performed semiquantitative RT–PCR assays for 12 ABC transporters shown to be highly expressed in root cells (Birnbaum *et al.*, 2003), including full-length and half-size transporters for all three treatments

(Fig. 7a,b). The results show that only one full-length ABC transporter (AtMRP2) was significantly upregulated on SA and MeJA treatment compared with the control (Fig. 7b). Other full-length ABC transporters, such as AtPgp1, AtPgp4, AtPDR6 and AtPDR7, showed no differential expression across all treatments compared with the control. In the group of half-size transporters, only AtNAP5 was upregulated by SA. AtGCN1, AtGCN3, AtATH6, AtATH10, AtNAP5 and AtTAP2 were downregulated significantly on NO treatment. The genes *AtATH10*, *AtNAP5* and *AtTAP2* were also downregulated significantly upon MeJA treatment.

## Discussion

We used a whole-genome approach to determine the genes specifically regulated in roots in response to exogenous SA, MeJA and NO. In this study, we focused on gene expression

related to two aspects: (1) defense responses in response to three signaling molecules; (2) the root-secreted phytochemicals and the possible transport systems involved in these processes. All three signaling molecules significantly (more than twofold up- or downregulated) altered gene expression for < 1% of the plant's genes; this result in roots is comparable with the gene expression studies reported on leaves (Parani *et al.*, 2004; Jung *et al.*, 2007; van Leeuwen *et al.*, 2007) (Fig. 1). On the whole, we did not find much overlap of the genes differentially expressed in response to the various signaling compounds (Fig. 4). This lack of overlap may be explained partly by the fact that the JA and SA pathways are mutually antagonistic, and treatment of the plant with JA resulted in inhibited expression of SA-dependent genes in *Arabidopsis* leaves (Thaler *et al.*, 2002). Therefore a similar antagonistic effect between SA and JA occurs in roots. Similarly, NO acts synergistically with various defense responses, but SA may antagonize the NO signaling pathway by scavenging (Hermann *et al.*, 1999). Moreover, salicylates inhibit the activity and transcript of induced nitric oxide synthase (iNOS) (Farivar & Brecher, 1996).

### Defense signaling in response to elicitation

All three signaling molecules expressed genes related to defense signaling/activation, but the genes induced or repressed by each signaling molecule were different (Tables S2–S4). This specificity was evident in other gene categories, including antimicrobial effectors, cellular redox state, transcription factors, metabolism and transporters. Jung *et al.* (2007) reported that 137 genes were significantly (more than twofold) up- or downregulated in response to MeJA treatment of rosette leaves of *Arabidopsis*; this number is very close to that found by our study, in which 125 genes were significantly (more than twofold) up- or downregulated in roots. The genes *VSP*, *JR2* (Rojo *et al.*, 1998) and *PDF1.2* (Penninckx *et al.*, 1998) are considered to be marker genes induced by in response to MeJA in *Arabidopsis* leaves (Jung *et al.*, 2007). In our study, we did not observe a significant induced expression of these marker genes. The observed differences might be due to the time point of collection of root samples for transcriptome analyses; concentration of MeJA used for elicitation in our study; or a differential response by roots to MeJA. We found other differences and similarities in altering the gene expressions in response to the signaling molecules. For example, the genes ACC oxidase (*At1g05010*) and myrosinase-binding protein (*At1g52030*) were repressed in our study (Table S3), but they are induced in leaves (Jung *et al.*, 2007) on treatment with MeJA. However, ERD1 (*At5g51070*) was induced by MeJA 3.2-fold and 4.6-fold in root and leaf transcriptome analyses, respectively (Jung *et al.*, 2007). In addition to these differences, we observed differential expression of genes in the families of peroxidases, heat shock proteins, zinc finger proteins, and genes involved in cellular redox state (Table S3). But none of these genes was

observed in leaf transcriptome analyses in response to MeJA treatment reported by Jung *et al.* (2007). We observed similar dissimilarities when we compared our results for signaling molecules SA and NO with previous studies in leaves (Parani *et al.*, 2004; van Leeuwen *et al.*, 2007). These results indicate that different organs of the plant (leaves and roots) respond differently to these elicitors.

Exogenous application to leaves of the three signaling molecules tested here has been documented to induce the expression of a number of genes (Farmer *et al.*, 2003; Parani *et al.*, 2004; van Leeuwen *et al.*, 2007; Jung *et al.*, 2007) and also to increase the accumulation of different families of secondary metabolites related to defense responses (Vázquez-Flota *et al.*, 1994; Doughty *et al.*, 1995; Brader *et al.*, 2001; Memelink *et al.*, 2001; Zhao *et al.*, 2005; Hendrawati *et al.*, 2006). Generally, the transcripts involved in the phenylpropanoid pathway are induced by MeJA and SA in *Medicago* cell-suspension cultures (Suzuki *et al.*, 2005) and in *Arabidopsis* in response to pathogens (Tan *et al.*, 2004; Bednarek *et al.*, 2005). By contrast, the transcripts involved in the phenylpropanoid pathway were downregulated significantly in our root transcriptome analysis by MeJA and NO treatments, but both up- and downregulation were observed in the SA treatment. Other pathways involved in the defense response, such as the glucosinolate and brassinosteroid biosynthetic pathways, were altered in SA and MeJA treatments. The gene *CYP79B2* (*At4g39950*), which encodes a key enzyme of indole glucosinolate biosynthesis, was downregulated in our transcriptome analysis in response to MeJA treatment, but was upregulated by MeJA treatment in *Arabidopsis* leaves (Mikkelsen *et al.*, 2000). Myrosinase-related components are shown to be induced by MeJA treatment of leaves of *Arabidopsis* (Sasaki-Sekimoto *et al.*, 2005; Jung *et al.*, 2007); these components are part of the myrosinase complex involved in hydrolysing the intact glucosinolates into toxic compounds such as isothiocyanates and nitriles. Interestingly, in our root transcriptome analyses these myrosinase-binding proteins were shown to be downregulated by MeJA treatment. The data reinforce our conclusion that the response of various biosynthetic genes depends on the organ examined. The biological implications of this differential expression based on signaling molecule might be related to the different biotic challenges faced by above- and below-ground organs.

It has been shown that signaling molecules, when applied exogenously or as a systemic signal, increase the accumulation of different families of secondary metabolites related to defense responses (Vázquez-Flota *et al.*, 1994; Doughty *et al.*, 1995; Brader *et al.*, 2001; Memelink *et al.*, 2001; Zhao *et al.*, 2005; Hendrawati *et al.*, 2006). We found that several phytochemicals secreted by the plants in response to the three signaling molecules did not coincide with transcript profiling data for genes involved in the biosynthesis of their metabolic pathways. We identified by NMR three flavonoids (KGR, KR and KRR) and one indole (MIC) that are found in root-secreted

phytochemicals and differentially regulated by the elicitors. KGR, KRR and MIC increased in all three elicitor treatments, and KR increased only in response to MeJA treatment (Fig. 6; Figs S3, S4a, b). Upregulation of biosynthetic genes was not found in the roots, even when the compound was found in higher quantities in root exudates based on elicitation. This apparent disparity could be because the upregulation of transcripts involved in these pathways occurs immediately after addition of the signaling molecule and is downregulated after 3 h, when we collected the roots for transcriptome analysis; or the relationship between the RNA transcript level and protein turnover may not be proportionate (Cho *et al.*, 2007); or increased exudation may not require increased biosynthesis, instead representing the release of stored metabolites from within the root (Frag *et al.*, 2008).

### Transcription factors and root exudation

We hypothesized that the increase in the root exudation of phytochemicals in response to elicitors is partly controlled by transcription factors, transporters and regulation of metabolic biosynthesis. In our transcriptome analyses, we observed five families of transcription factors that were significantly differentially expressed in all three treatments (Tables S2–S4). Among these transcription factor families, several NAC members were upregulated at different levels by some elicitors. Specifically, the NAC transcription factor (At3g29035, AtNAC3) was upregulated by all three treatments, suggesting a common regulatory role for this transcription factor in the plant root's response to all three signaling molecules. The closest homologue to AtNAC3 is AtNAC2, which is expressed mainly in roots (overall 67% identity; 80.1% identity with the NAC domains) and is involved as a downstream component in ethylene- and auxin-signaling pathways and also in salt stress and lateral root development (He *et al.*, 2005). Other transcription factors involved in abiotic stress, such the WRKY, AP2, and bZIP families, were upregulated by some elicitors. Van der Fits and Memelink (2000) reported that jasmonate induced the AP2 domain transcription factor ORCA3, which regulates terpenoid indole alkaloids in the leaves of *Catharanthus roseus*. In our transcriptome analysis, AP2 domain transcription factors were upregulated by SA and NO, but no significant differential expression of these genes was observed with MeJA treatment. By contrast, MYB transcription factors, which are involved in regulating flavonoid biosynthesis (Grotewold, 2005; Galis *et al.*, 2006), were downregulated by only some treatments. Interestingly, no significant differential expression in response to MeJA was observed in the members of the AP2 domain transcription factor family in our root-focused study, but upregulation of members of this family were observed in leaf responses to MeJA (Jung *et al.*, 2007); additionally, none of the members of this family show differential expression in response to NO (Parani *et al.*, 2004) in leaves of *Arabidopsis*. It is possible that

some NAC transcription factors may play a role in root secretion of phytochemicals by positively or negatively regulating the metabolic pathways, expression of transporters, or other genes involved in this process.

### Possible transporters involved in the root exudation process

In all three signaling molecule treatments, we observed transporters for sugars, amino acids, cations and other molecules (Tables S2–S4) to be differentially expressed. The majority of the transporters were upregulated by SA and MeJA, but downregulated in NO treatment. Specific transcripts for the MFS and MATE transporters were differentially expressed in response to SA and MeJA treatments, providing evidence for the possible implication of these transporters in root secretion. Interestingly, we did not observe any significant differential gene expression of ABC transporters (more than twofold up- or downregulated) in response to three signaling molecules in transcriptome analysis, although recent reports have shown that ABC transporters are partially involved in root exudation processes (Loyola-Vargas *et al.*, 2007; Sugiyama *et al.*, 2007; Badri *et al.*, 2008). To improve the resolution of this analysis, we performed semiquantitative RT–PCR to look for the differential expression of ABC transporters highly expressed in specific root cells (Birnbaum *et al.*, 2003; Badri *et al.*, 2008) in response to these signaling molecules. We found that only a few ABC transporter genes were upregulated by the signaling molecule treatment in RT–PCR analysis: AtMRP2 was upregulated in response to SA and MeJA treatments, and AtNAP5 in response to SA treatment (Fig. 7b). Parani *et al.* (2004) reported that AtMRP2 was upregulated by NO treatment in *Arabidopsis* leaf tissue, but in our analysis no significant differential expression was observed in root tissues, again indicating that the aerial and root tissues respond differently to this signaling compound. Further, in our exudate profile analysis we found that peaks P11 (MM 308) and P12 (MM 326) increased only in response to SA treatment compared with the control; the upregulation of AtNAP5 by SA treatment in the RT–PCR assay provides evidence for a possible role for AtNAP5 in secreting these compounds from the root cells (Fig. S4). Conversely, we found that peak 9 increased in our exudate profiles (Fig. S4) on NO treatment, but the majority of the transporters were found to be downregulated in both transcriptome and RT–PCR analyses. The lack of correlation might have the following causes: increase in biosynthesis of the compound (peak 9) inside the root tissue on NO treatment may not need to induce the expression of transporters in order to transport outside the cell; secretion of the compound (peak 9) from the cell may happen via a passive process and may not require active transport; or there may be a lack of identification of the compound (peak 9) and the transportation process is unexplainable.

We did not observe any ABC transporters significantly differentially expressed (more than twofold up- or downregulated) in the transcriptome analysis of any of the treatments, but we found differences in their expression in RT-PCR analysis. Possibly the change in the expression of any ABC transporters in RT-PCR analysis is due to the threshold level of significance considered in transcriptome analyses (a gene must be more than twofold up- and downregulated to be considered). In RT-PCR assays, the differential gene expressions of ABC transporters are significant at a  $P$  value  $< 0.05$ , but the fold induction is less than twofold the control, except AtNAP5, which shows a 4.2-fold induction compared with the control (Fig. 7b). However, we observed the same trend in both transcriptome and RT-PCR analysis. The expression of some PDR family members, such as PDR12, were strongly induced by SA, MJ and ethylene in *Arabidopsis* leaves (Campbell *et al.*, 2003), but in our analysis none of the PDR genes showed induced expression. This result could be due to the fact that the ABC transporter genes chosen in our study were highly expressed in roots, and that PDR12 showed no expression in roots, although it is reported in leaves (van den Brûle & Smart, 2002).

In conclusion, we found that there is not much overlap observed in the transcriptome analyses in root responses to each of these signaling molecules, indicating that they act through different signaling mechanisms; these signaling molecules alter gene expression depending on the plant organ (root or leaf) to which they are applied; and signaling molecules alter secretion of root-exuded phytochemicals by regulating transporters (ABC, MATE, MFS), transcription factors and metabolic biosynthesis. Further studies are needed to dissect the specific genes identified in this manuscript involved in cross-talk of the three signaling molecules, mediated defense responses, biosynthesis, transport and regulation of secondary metabolites, and how they relate to root exudation.

## Acknowledgements

This work was supported by a grant from the National Science Foundation (to J.M.V. and F.R.S.; MCB-0542642). V.M.L.-V. is a recipient of scholarship from CONACYT, Mexico. We are grateful to Emily Wortman-Wunder for editorial assistance and the members of Jorge Vivanco's laboratory for helpful discussion.

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## Supplementary Material

The following supplementary material is available for this article online:

**Fig. S1** Name and structures of the compounds identified by nuclear magnetic resonance from *Arabidopsis* root exudates.

**Fig. S2** Verification of microarray-based gene expression changes by quantitative RT–PCR.

**Fig. S3** Chromatogram of representative HPLC/MS *Arabidopsis thaliana* plant root exudate profiles in the presence of 250  $\mu$ M of elicitor after 6 h treatment.

**Fig. S4** Height of the major peaks from the exudates of *Arabidopsis* roots in the presence of signaling molecules.

**Table S1** List of primer pairs used in this study

**Table S2** Select list of genes differentially expressed in *Arabidopsis* roots in response to salicylic acid (250  $\mu$ M) treatment for 3 h

**Table S3** Select list of genes differentially expressed in *Arabidopsis* roots in response to methyl jasmonate (250  $\mu$ M) treatment for 3 h

**Table S4** Select list of genes differentially expressed in *Arabidopsis* roots in response to nitric oxide (250  $\mu$ M) treatment for 3 h

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